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## **EFFECTS OF A NUTRIENT-ENRICHED BEVERAGE ON HOST DEFENSE MECHANISMS OF SOLDIERS COMPLETING THE SPECIAL FORCES ASSESSMENT AND SELECTION SCHOOL**

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ASSESSMENT AND SELECTION SCHOOL

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We evaluated a liquid supplement containing anti-oxidants, indigestible carbohydrate, structured lipid, and vitamins and minerals for its effects upon the immune responses of soldiers participating in the US Army Special Forces Assessment and Selection School. Subjects on the experimental supplements as well as controls lost approximately 6.4 pounds in 3 weeks with an estimated daily expenditure of 5040 Kcals. The in vivo measurement of cellular immune function, delayed type skin hypersensitivity at exit, suggested that subjects in the treatment group had a greater response ( $p = 0.07$ ) and fewer subjects in the treatment group were anergic (18% vs 39%). The pattern of change in white blood cells and lymphocyte numbers and subsets were consistent with increased risk of infection during training with some attenuation of these effects with the supplement. This study provided a comprehensive evaluation of immune function and yielded evidence supporting the hypothesis that specific nutritional supplements may enhance immune function, especially under stressful conditions or scenarios.

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## EXECUTIVE SUMMARY

Researchers have documented the relationship between malnutrition and disease susceptibility for many years. This has resulted in the examination of the impact of nutrition on immune function. Ross Products Division/Abbott Laboratories and the U.S. Army Research Institute of Environmental Medicine have collaboratively investigated potential benefits of nutrient supplementation and nutritional strategies to minimize potentially harmful immunologic changes in a variety of conditions. In the present study we tested the ability of a ready-to-feed liquid supplement containing antioxidants, structured lipid (made from canola and medium chain triglycerides), vitamins, minerals, indigestible carbohydrate (fructooligosaccharide), carnitine and taurine, to determine its influence on minimizing potentially negative immunological changes found in soldiers participating in United States Army Special Forces Assessment and Selection School (SFAS). We compared their immune responses to another group of soldiers that consumed an isonitrogenous, isocaloric control product. This study was also conducted to characterize the physiological, nutritional and immunological effects of SFAS training and help define the parameters that are most useful in denoting significant changes of status in SFAS.

Two hundred soldiers participating in a Special Forces Assessment and Selection Course conducted by the United States Army John F. Kennedy Special Warfare Center and School, Fort Bragg, North Carolina volunteered to participate in this prospective, randomized, blinded, placebo controlled study. The test group (n=100 soldiers) was designated to consume their regular diet consisting mainly of meals ready-to-eat (MRE) plus a ready-to-feed treatment product (8 oz., two times per day) containing a similar nutrient composition to Ensure Plus\_ with added levels of antioxidants (all natural vitamin E, vitamin C,  $\beta$ -carotene), minerals (selenium, zinc and copper), structured lipid (from canola oil and medium chain fatty acids), carnitine, taurine, and indigestible carbohydrate (fructooligosacchride). The control group (100 soldiers) consumed a ready-to-feed product of similar taste and appearance containing similar amounts of macronutrients and energy. Dietary intake, body weight change, and immune function were measured before and after the physically and psychologically demanding 21 day training course.

There were 50/100 and 57/100 soldiers that completed SFAS in the treatment and control groups respectively. Dropping from the course was primarily due to administrative, voluntary withdrawal or board withdrawal. Rates were similar to previous courses in previous years. There were no differences between drop rates and reported intolerance to either product. Subjects lost approximately 2.9 kg in 3 weeks. Combining the dietary intake with the weight loss data it was estimated that soldiers had an energy expenditure of 5,040 kcals per day.

The *in vivo* measurement of overall cellular immune function, delayed type-skin hypersensitivity-DTH at exit (total induration), suggested that subjects consuming the treatment



product had a greater response ( $P=0.07$ ). Similarly, there were fewer subjects in the treatment group compared to the control group that were DTH anergic (18% vs 39%) to the skin test, suggesting a lower risk for infections in the treatment group. The percentage of soldiers found in the control group who were anergic was consistent with previous studies in SFAS. By contrast there was a trend toward increased lymphocyte responsiveness to mitogen stimulation from soldiers fed the treatment product. Several changes were noted in lymphocyte subsets during the course of the study. The pattern of change in white blood cells, lymphocyte numbers and subsets was consistent with increased risk of infection within SFAS, and the treatment product attenuated some of the changes. Cell profile changes in the treatment group also seemed to indicate that neutrophils may be migrating to the target or affected tissues (skin or lungs) rather than in the blood. Maintenance of lymphocyte populations such as cytotoxic/suppressor, Th1 lymphocytes, and neutrophils was found in the treatment-formula-fed group. A number of lymphocyte populations correlated with DTH responses; however, there was no consistency between control and treatment fed groups. This finding implies that the groups responded differently and that immunologic function was influenced by the treatment formula. Overall, the treatment formula appeared to minimize or attenuate the immunologic changes associated with SFAS training (strenuous physical and psychological stress).

An understanding of the immunologic effect of sustained stress provides an opportunity to study nutritional and immunologic mechanisms which are involved. It is recommended that the study formula be used to design other food forms (ie. bars) that can be eaten by soldiers under most operational conditions. In conclusion, this study provided a comprehensive evaluation of immune function and yielded evidence supporting the notion that nutritional components in the treatment product provided immunologic benefit to individuals under stress.

## INTRODUCTION

Historically, researchers have documented the relationship between malnutrition and disease susceptibility for many years. This observation has resulted in the examination of nutrition's impact on immune function and led researchers to evaluate the impact of deficiencies of specific nutrients on immune function. Ross Products Division/Abbott Laboratories has investigated potential benefits of nutrient supplementation and nutritional strategies to minimize immunologic changes in a variety of conditions. The difficulty has been in identifying models or clinical scenarios where immunologic dysregulation occurs and an opportunity to intervene with nutrients. One promising situation which has been evaluated collaboratively by the USARIEM and Ross Products Division is military training.

The United States Army Special Forces Assessment and Selection Course (SFAS) is highly demanding both physically and emotionally (21 days duration). Earlier studies documented a negative energy balance (1,379 kcals/day) in soldiers who participated in the course; however there were no significant clinical manifestations of vitamin and/or mineral deficiencies. One observation from that study was a reduced lymphocyte proliferation response (reduced on average 23%) to mitogen stimulation. This observation suggested that multi-stressors (negative energy balance, sleep deprivation, physical activity, and psychological stress) may have negative effects on immune function and the ability of lymphocytes to combat infections. The SFAS scenario provides a unique population to study because the soldiers have similar environmental factors (including diet and physical stress) and suppressed immune function and higher rates of infection than among non-stressed soldiers. Interventional nutrition studies with this group allow an evaluation of the effects of nutrients on immune function (suppressed in previous studies). Furthermore, military training scenarios permit the evaluation of a large number of subjects in a short period of time and focuses on the methods required to identify immune dysregulation.

To establish whether the immune changes observed during military training influence infection rates or non-battle injuries, it is valuable to review findings from studies conducted during military conflicts. For example, during the last four major military conflicts that have involved U.S. soldiers, disease and non-battle injuries (DNBI) have accounted for significant loss of manpower (64). During World War II the ratio of Navy and Marine Corps hospital admissions for DNBI and combat-related wounds and injuries was 88:1. It was also found that in Marine Corps personnel serving in Vietnam, for every 100 men wounded in action, there were 128 men who were hospitalized for DNBI (64). Exposure to pathogens from environmental factors and crowded living quarters contribute to disease susceptibility of military units even during routine peace time operations. Seay et al (77) found that after a 10 day port visit to Rhodes, Greece by the crew of the USS Forrestal, 777 cases of gastroenteritis were reported. This accounted for 15% of the work force and a cost of 462 man-days of lost work. Major Wallace J. Seay stated, "... lost duty days, and the burden of providing medical confinement may be more critical than fatal illnesses. Fatalities may be replaced but sick soldiers continue to occupy positions,

decrement performance, and consume large quantities of medical supplies (77, pp. 3-4)." While there are many factors that may contribute to susceptibility to infection and disease during military operations, the effects of high levels of both physical and psychological stress undoubtedly have a significant impact on host defense mechanisms. Historically, few opportunities have existed to conduct well controlled field research to study the effect of combat training on immunological responses. It is also recognized that nutrition is an important factor that can be manipulated to minimize immune dysregulation during stressful situations.

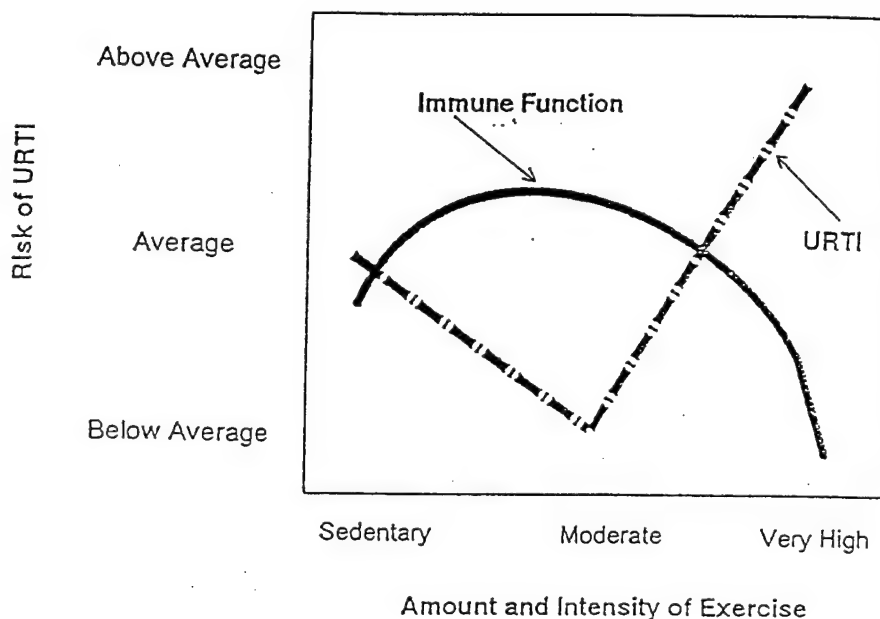
It is therefore not surprising that nutritional inadequacies result in immune dysregulation and can profoundly influence susceptibility to infection. Cells of the immune system require a variety of nutrients for proper function. Furthermore, nutrients are essential and play a vital role in enzyme synthesis and activity required for normal immune function, e.g. many kinases and transferases require zinc. For example, it is not uncommon to observe diminished immune response (anergy) to DTH in individuals with severe malnutrition. The influence of mild malnutrition on immune regulation and an understanding of specific levels of nutrients for optimal nutrition remain to be investigated. Therefore, we have utilized a unique military training scenario, SFAS (similar to combat stress) in which a nutritional formula was tested to determine the possible immunologic impact of stress and nutrition.

Initially we investigated the immunologic effect of the nutritional supplement glutamine (15 grams/day) given to soldiers participating in SFAS. Supplemental glutamine was shown to influence immune cells' responsiveness in individuals recovering from traumatic injury (9, 18, 81). Glutamine also reduces infections in marathon athletes (17) and prevents the suppression of lymphocyte responsiveness to mitogen following exercise in rats (58). However, we found that supplemental glutamine did not attenuate the reduction in lymphocyte proliferation seen after SFAS training (unpublished). We therefore examined an antioxidant-enriched beverage supplement containing  $\beta$ -carotene, vitamin C, and all natural vitamin E as well as selenium for influence on immune function. All of the components were shown to be beneficial for cellular immunoresponses (27, 33, 40, 68). The present study provided evidence that the supplemental antioxidant mixture attenuated the reduction of lymphocyte proliferative responsiveness during SFAS training. Furthermore, this study confirmed that the SFAS course induced immune suppression (DTH and lymphocyte responsiveness to mitogen) and proved to be a model to determine the influence of stress on immune function and host defense mechanisms, and evaluate whether nutritional supplementation could influence immune function.

The idea that high intensity training might reduce immune function was first postulated in 1932, based on the observations that muscular fatigue predisposed individuals to infections, especially respiratory pathogens (2). Several epidemiological studies have documented an increased incidence of upper respiratory tract infections (URTI) following strenuous exercise (62, 70). Conversely, it appears that moderate exercise is beneficial in the defense against disease: 1) persons performing moderate exercise have lower risk of infection than sedentary controls (34, 61) and 2) mice undergoing moderate exercise training have reduced susceptibility to bacterial and protozoan infections compared to sedentary controls (16, 19). Therefore, it appears that

moderate exercise can convey enhanced immunological responses and more chronic and severe stress, as seen in SFAS, leads to a decreased immune function and increased susceptibility to environmental pathogens (Figure 1). Understanding the influence of such stressors on specific immune responses is critical to improving the performance and sustainability of the deployed combat forces. The SFAS scenario allows evaluation of the effects of nutrients on immune responsiveness in healthy young adults experiencing a temporary suppression in immune response under the influence of a multi-stress environment.

Figure 1. Relative risk of URTI with intensity of training (adapted from 61)



Soldiers in SFAS are subjected to energy and sleep deprivation, mental stress, and intense physical exertion. Stress alters both innate and antigen specific elements of the immune system. The specificity of the immune system is a result of antigen-cell interaction and the integrated function of specific lymphocyte subsets. Immune effector cells, B lymphocytes (B cells), natural killer lymphocytes (large granular lymphocytes), and T lymphocytes (T cells) interact to mount a defense against invading pathogens or abnormal cells. In a very simple view, B cells form the humoral or antibody production arm of specific immunity while T cells are associated with cell-mediated immunity. T cells, in addition to their role as "killer cells," have important functions in regulating B cell functions and in "directing" cellular trafficking in the development of highly specific responses. In a clinical setting numerous laboratory tests can be performed to evaluate immune function. Table 1 contains examples of possible laboratory tests and methods to evaluate immune function.

Table 1. Examples of Laboratory Tests of Immune Function.

White Cell Numbers

- Automated cell count (complete blood count)
- Flow cytometry (cell population analysis by immunophenotype analysis)

Cell Function

- Lymphocyte proliferation following mitogenic stimulation
  - \_ Tritiated thymidine uptake
  - \_ CD69 expression
- Cytotoxicity
  - \_ NK cell cytotoxicity
  - \_ Lymphokine activated cytotoxicity
  - \_ Antigen specific CD8+ cytotoxicity
- Cytokine production
- T- and B-lymphocyte cooperation
  - \_ CD40 ligand expression
- Immunoglobulin production
  - \_ Response to vaccination

In Vivo Immunity

- Cutaneous reaction
  - \_ Delayed type skin hypersensitivity
- Resistance to viral infections
  - \_ Rhinovirus challenge
- Incidence of infectious disease

*In vitro* tests alone do not give a complete indication of clinical status. Furthermore, *in vitro* studies need to be performed on fresh samples and a single test may not be indicative of overall immune system effectiveness. Therefore, when evaluating immune function it is important to evaluate various components of the immune response. It is also important to relate the test indicators to overall immune status and risk of infection. Examples of *in vivo* immune status markers include: DTH, infection rate, or response to pathogen or vaccine. Experience with DTH over the last several years has shown good correlation between lack of skin reactivity (anergy) and measured risk of infection and mortality (21, 78). Reduced cell mediated immunity can limit development of an effective immune response against intracellular pathogens, including viruses, bacteria, fungi, and protozoa. In this regard we have found that 30-50% and 50-70% of the soldiers participating in SFAS and Ranger Training respectively have suboptimal DTH responses following training compared to approximately 1-3% in the general population.

Another test used to evaluate immune function in SFAS trainers involves measuring the proliferative response of peripheral blood lymphocytes following exposure to stimulant or

mitogen and requires that lymphocytes incubate in culture for several days. Another method is to quantitate cell populations, using flow cytometry and monitor the expression of a key cell surface protein, CD69. The method uses monoclonal antibodies (tagged with fluorochromes) directed against these cell surface proteins. By this method, cells do not need to be cultured for longer than 4 hours and cells are stimulated in their environment, whole blood, with minimal alterations.

In this study we examined the effects of a multi-nutrient supplement on immune cell function. This vitamin and mineral nutrient supplement contained protein, carbohydrates, indigestible carbohydrate, antioxidants, and structured lipids. We studied a number of parameters including delayed type hypersensitivity and evaluated peripheral blood samples for changes in cellular populations, and cellular function. It is important to point out that DTH is used as a predictor or indicator of disease susceptibility. An adequate DTH response requires several distinct steps, e.g. antigen uptake, processing, and presentation to effector cells. The proper functioning of these steps are required for the expression of a quantifiable immune response pattern. DTH is somewhat imprecise; however, variability and biases are minimized by administering and reading the skin reaction in a standardized fashion and conducting the study in a blinded design.

## **OBJECTIVES**

The objectives of the study were:

1. To test the ability of a control (isonitrogenous and isocaloric ready-to-feed) or novel nutritional ready-to-feed supplement (containing previously tested formula of antioxidants) plus increased energy supplement (containing structured lipid-canola and MCT oil), vitamins, minerals and indigestible carbohydrate to minimize or prevent immunological changes seen in SFAS.
2. To further characterize the physiological, nutritional and immunological effects of SFAS training; with the goal of determining the parameters that are most useful in defining significant changes in immune cell function in SFAS (will follow in part 2 of this report).

## **MATERIALS AND METHODS**

**SUBJECT SELECTION** All aspects of this protocol were approved by the United States Army Research Institute of Environmental Medicine's (USARIEM) Human Use Review Committee in accordance with current military standards. Soldiers in the Special Forces Assessment and Selection course, conducted by the United States Army John F. Kennedy Special Warfare Center and School, Fort Bragg, North Carolina volunteered to participate in this study. Two classes (April 1996 and Oct 1996) were recruited (approximately 100 soldiers per group). Males were the only participants because Special Forces School is not currently open to women. Volunteers were briefed on the purpose of the study and of its potential risks and benefits. The soldiers were further informed that they could withdraw from the study at any time without penalty and that their participation or withdrawal would not influence their ultimate selection to the Special Forces Qualification Course. The principal investigator for this study briefed the



prospective volunteers' unit commanders on their responsibility to ensure that consent to participate in this research project was voluntary. The study (Table 2) was described to the soldiers and volunteers signed Informed Consents and a volunteer Registry Data Sheet prior to participation.

Table 2. Course description of SFAS training.

Day 1-6	Day 7-11	Day 12-13	Day 14-19	Day 20-21
In-process	Military	Log Drill	Situation and	Selection
PT Test	Orienteering	General Subjects	Reaction Stakes	Out-
Swim Test	Battle March		Long Range	Processing
Rucksack			Movement	
Marches				
Obstacle Course				

After recruitment the test subjects were randomly assigned to an isonitrogenous and isocaloric control product or the treatment product. The treatment product contained the nutrient composition of Ensure Plus with additional elevated levels of antioxidants (all natural vitamin E, vitamin C,  $\beta$ -carotene), minerals (selenium, zinc and copper), structured lipid (from canola oil and medium chain fatty acids), carnitine, taurine, and indigestible carbohydrate (fructooligosaccharide). Products were made to be similar in appearance and taste. Therefore, this was a prospective, double blind, randomized, controlled trial of supplemental ready-to-feed products. The beverages were provided in an easy open 8 ounce can and were consumed twice daily (control=EN 9603 and treatment=EN 9604). Both beverages supplied approximately 360 kcals/8 oz. All volunteers were allowed their normal rations, a combination of hot meals and field rations (Meals, Ready-to-Eat, MRE's).

**PRODUCTS** Ready-to-feed liquid products were formulated and manufactured by Ross Products Division of Abbott Laboratories. The nutritional products were isonitrogenous and isocaloric (control and treatment). The nutrients included in the treatment product supplied concentrations that potentially may be beneficial under stressful conditions. Some of the nutrients included in the treatment product have been found to strengthen immune function. A brief summary of the individual ingredients with accompanying rationale for inclusion will follow.

We found inadequate energy intake and weight loss in previous SFAS studies. It is difficult to determine whether increased intake of energy or specific nutrients would adverse prevent immunologic changes. In this study each product provided protein, fat, and carbohydrate as 14%, 30% and 56% of calories, respectively. We also included the same amount of energy and nitrogen in the control, ready-to-feed product to prevent any confounding factors. Both products contained similar proteins (high biologic value-casein and soy) and carbohydrates (sucrose and maltodextrins). Most dietary fats are triacylglycerol compounds with glycerol esters of fatty acids

of 12-18 carbon atoms. The treatment product contained a novel structured lipid made of medium chain (MC) triglycerides (6-8 carbon atoms) and canola oil (long chain fatty acids-LCFA) (16-18 carbon atoms). This novel fat has random placement of the medium chain and long chain fatty acids on the glycerol backbone. Studies of structured lipids have been conducted in enteral and parenteral nutritional regimens and have been shown to beneficially affect immune function in surgical (47), burn (3), and trauma (41) patients.

Fat digestion begins at ingestion, but the majority of digestion and absorption occurs in the small intestine. Fat is released into the small intestine more slowly than water miscible substances. Fats, particularly LC triglycerides, are emulsified by bile salts. This allows dispersion so that pancreatic lipase can hydrolyze the triacylglycerol into fatty acids and transport 2-monoacylglycerol which is then re-esterified upon absorption and transport. Fat digestion and absorption changes markedly when fatty acids that contain fewer than 12 carbon atoms are consumed. These fatty acids are digested, absorbed, and utilized even in the presence of pancreatic insufficiency. Therefore, by having MC as well as long chain fatty acids on the same glycerol molecule we hoped to increase absorption and utilization of fatty acids as an energy source. This may minimize catabolism of muscle tissue and muscle glycogen thereby improving performance, decreasing muscle injury and reducing muscle injury-derived inflammatory factors. MCTs alone aren't used because once they are consumed, they are absorbed into the portal circulation and metabolized immediately by the liver; whereas LCT are absorbed and transported via the lymph and then put into circulation. Upon delivery to the cells there is a difference between LC and MC fatty acid utilization, particularly as energy sources in the mitochondria. Current data suggests that MC fatty acids are oxidized preferentially over long chain fatty acids (76). We attempted to shift energy utilization away from muscle glycogen or protein and allow MC fatty acids to be utilized as muscle energy sources.

Carnitine was included in the treatment product because it is required for lipid metabolism and is crucial for energy flow within skeletal muscle. Trauma and infection lead to loss of carnitine (88). It has been shown that carnitine supplementation in rats decreases morbidity and mortality when challenged with lipopolysaccharide (LPS). Carnitine also improves dysregulated lipid metabolism in septic and cachectic rats (92). A recent review described role of carnitine in the immune system as enhancing: 1) phagocytosis by granulocytes, 2) PHA induced T-lymphocyte activation, and 3) the membrane-activating effect on human NK cells (86).

Strenuous exercise has been shown to cause increased production of oxygen free radicals (80). SFAS is demanding and highly stressful, both physically and mentally. If free radicals are produced without adequate metabolic control, tissue damage and immune dysfunction may result. Cells such as polymorphonuclear leukocytes, macrophages and lymphocytes carry out many functions which generate free radicals. Accumulation of free radicals can result in tissue injury, delayed wound healing and increased susceptibility to infectious diseases (28, 75). Immune cells contain concentrated amounts of antioxidants (59), and function most efficiently when adequate levels of antioxidants are present (43).



$\beta$ -carotene is a vitamin A precursor; however, at elevated doses it is not toxic unlike other retinols (vitamin A) (4, 37).  $\beta$ -carotene is an effective chain-breaking antioxidant due to its ability to trap peroxy radicals. It seems to be a unique antioxidant that is most effective at low atmospheric pressures as well as efficient in quenching singlet oxygen species (12, 13). Gottlieb et al. (29) found that  $\beta$ -carotene is effective in reducing lipid peroxidation (malondialdehyde [MDA]) in serum. Several studies have demonstrated that  $\beta$ -carotene enhances immune function independent of its vitamin A activity (5). One human study evaluated immunologic effects of supplementation with 15-300 mg  $\beta$ -carotene. It was found that daily supplementation of  $\beta$ -carotene prevented cell mediated immune suppression as measured by DTH response (45). It was found in another study that when healthy male smokers were given 20 mg of  $\beta$ -carotene for 14 weeks, there was a 12% higher proliferative response to the mitogen phytohemagglutinin (PHA) than in a placebo treated group. Watson et al. (90) found that 2 months of  $\beta$ -carotene (15-60 mg) supplementation enhanced immune responses in elderly subjects. Lastly, a study involving *in vitro* culture of human PBMC found altered phenotypic changes and enhanced activation of cells when incubated with  $\beta$ -carotene (71). Kanter et al. (45) demonstrated in humans that an antioxidant mixture of 592 mg of  $\alpha$ -tocopherol, 1000 mg of ascorbate, and 30 mg of  $\beta$ -carotene, decreased markers of lipid peroxidation (MDA and breath pentane) after running to exhaustion.

Vitamin E is a fat soluble vitamin found in membranes throughout the body and is regarded as an effective lipid, chain-breaking antioxidant. In rats, during exhaustive exercise, the markers of oxidative stress are usually isolated from biopsied muscles. Davies et al. (22) found that vitamin E deficient rats manifested increased susceptibility to free radical damage. This susceptibility caused premature exhaustion, greater fragility of lysosomal membranes and decreased muscle mitochondrial respiratory function. Vitamin E supplementation (1000 IU for 10 days) significantly reduced breath pentane output (a marker of oxidative status and stress) in healthy adults consuming a normal, mixed diet (53). Vitamin E has been proposed to increase longevity by influencing the immune system (32). Immune cells contain high concentrations of vitamin E (32). In studies wherein humans were supplemented with 1200 IU vitamin E for 2 weeks during strenuous physical exercise there was a significant reduction of breath pentane (25, 26). Animal studies have demonstrated that vitamin E deficiency causes immune suppression (7, 33, 84) and once the vitamin E deficiency is corrected, immune responses return to normal (85). Only a few studies have been performed in humans to evaluate the effects of vitamin E supplementation on immune function. Meydani et al. (55) supplemented the diets of elderly individuals with 800 IU of vitamin E. After 30 days of supplementation, there was an enhancement of DTH response and lymphocyte proliferation after mitogenic stimulation. Later Meydani et al. (56) reported that supplementation with vitamin E (60, 200, 800 IU) to elderly subjects for 235 days improved DTH and increased antibody titer to hepatitis B vaccine. Prasad (72) treated 18 young males with 300 mg vitamin E. After three weeks of supplementation, there was an increase in bactericidal activity of peripheral blood leukocytes and cell mediated immunity. In the treatment ready-to-feed product we used all natural (RRR- $\alpha$ -tocopherol) vitamin E. It has been shown to be 1.4 times more biologically potent than synthetic (racemic) forms (14, 38).

Vitamin C is a water soluble antioxidant with strong reducing potential. It scavenges superoxide radicals, hydroxyl radicals, peroxy radicals, hydrogen peroxide and single oxygen. Like vitamin E, vitamin C has been found to reduce the effects of oxidative damage. Kaminski and Boal (44) noted that 3000 mg of vitamin C for 3 days reduced muscle soreness. Studies that link ascorbic acid (AA) to immune status do so through AA's antioxidant properties. Several laboratories have studied the effect of AA on the physiology of white blood cells and prevention of oxidative damage by free radicals. The concentration of AA in leukocytes is approximately 80 times greater than that found in plasma (1, 35) suggesting an important role in the function of leukocytes. *In vitro* studies suggest that AA may enhance human cellular immune function (23, 65). Generally, AA has been shown to affect the functions of leukocytes, and macrophages as well as wound healing, and allergic reactions.

Selenium is an integral component of the enzyme, glutathione peroxidase, and catalyzes the breakdown of peroxides. Selenium deficiency causes an elevation in pentane production in rats (26). Thus, selenium, although not an antioxidant, plays an essential role in preventing oxidative damage. The metabolic function of selenium with respect to immune function has been reviewed (48). Peretz et al. (69) supplemented institutionalized subjects with 100 µg selenium/day for six months. The group supplemented with selenium demonstrated improved lymphocyte proliferative response to mitogen. In another study, researchers supplemented diets with 200 µg of selenium daily for three weeks and observed enhanced NK cell cytotoxicity (27). Greenman et al. (31) found that polymorphonuclear leukocyte phagocytic function was enhanced by the ingestion of 182.5 µg of selenium per day. These results were similar to findings in animal experiments (50, 83). Therefore, it is generally accepted that selenium is necessary for optimal immune function, and in our study was supplied in the supplement at a level of 100 µg/day. It should be pointed out that unlike the other nutrients that were supplied in the treatment product, selenium has a narrow range for optimal health. Toxic levels occur following supplementation with between 1500-5000 µg/day (51). The concentration in the treatment was far below the toxic level, and we believe that the optimal level is between the RDA (70 µg/day) and 400 µg/day.

Zinc is an important cofactor for many antioxidant enzymes, including lactate dehydrogenase and superoxide dismutase (20, 24). Many investigators have reported that zinc deficiency adversely affects immune function. Pariza (66) observed that zinc deficiency caused a 25% loss of body weight in young adult mice and resulted in a 50% loss in thymic weight. The antibody and cell-mediated immune responses were also about half the level of normal mice. Several studies have evaluated selenium and zinc status during stress. Singh et al. (79) noted that blood levels of both minerals were significantly reduced during US Navy SEAL training despite adequate intakes (met MRDA) for these minerals. Miyamura et al. (57) found that during a 34-day intensive training exercise (US Army) there was a depressed serum zinc level as well as elevated urinary zinc excretion. Chen (20) found that zinc deficiency in mice could lead to increased free radical production which was exacerbated by exercise.

Taurine (a β-amino-sulfonic acid) appears to be an effective scavenger of peroxidation products, is important for immune function (91), and was included in the treatment product.

Recently, Stapleton et al. (82) reviewed the influence of taurine on host defense. They demonstrated that taurine plays a pivotal role in many physiologic functions (ie. osmotic regulation, antioxidation, detoxification and stimulation of glycolysis and glycogenesis), and it influences neutrophil and macrophage function. Taurine constitutes greater than 50% of the free amino acids in lymphocytes (82). Lymphocytes seem to have a high dependence on  $\beta$ -amino acid transport and calcium flux (67). Furthermore, other types of stress or injury such as surgery (60), cancer (30) and sepsis (63, 89) are correlated with decreased levels of plasma taurine.

Lastly, it is important to note that we are supplementing the diets of soldiers during SFAS as a means of evaluating whether nutrient supplementation may benefit the soldier during intense stress. We do not believe that clinical deficiencies are developing or manifestations of vitamin or mineral deficiencies are occurring during or as a result of SFAS. The intense training may, however, increase requirements of various nutrients above the Military Recommended Dietary Allowances (MRDA). Providing optimal levels of nutrients should reduce illness, injury, or risk of infectious diseases during stress. We have chosen modest levels of nutrients that have the potential to affect the immune response without jeopardizing safety. This combination of nutrients more closely approximates an individual's diet as nutrients seldom act alone in regulating biochemical and metabolic processes.

**DIETARY INTAKE** Food intake data was collected using 24-hour dietary records in which each subject recorded daily food and fluid intake. Self recorded intake methods have produced accurate results (15). Each subject was instructed and trained in accurately completing the daily food records. Trained data collectors reviewed records with the volunteers daily to ensure that they were completed correctly. On Days 11-13 subjects were served "A" rations at a dining facility at Camp Mackall while MRE's comprised the remainder of their food intake. A record of individual intakes was taken at each meal using a visual estimation technique (73). The gathering of dietary intake data allowed an assessment of the caloric and nutrient intake during the course. Food records were evaluated for nutrient composition using the field ration nutrient database provided by the US Soldier Systems Center - Natick. Mean nutrient intakes were compared to the MRDA (Army Regulation 40-25, 1985).

**BLOOD COLLECTION AND ANALYSIS** Blood samples were collected in five separate vacutainers by trained phlebotomists between 0400 and 0600 hours at baseline (before supplementation) and at the end of the study (Day 19). The total amount of blood drawn for the study was approximately 70 mL.

<u>Tube</u>	<u>Analysis</u>	<u>Rationale</u>
Red Top (Serum Tube)	Retinol, tocopherol, vitamin C, albumin, alkaline-phosphatase, BUN, SGPT, SGOT, and triglycerides	Measurements of key nutritional markers
Royal Blue Acid-Washed	Lymphocyte blastogenesis	Measures ability of immune

Sodium (Heparin Tube)		cells to respond to mitogen.
Purple Top (EDTA Tube)	Complete blood count	Determine population of cells in the blood
Purple Top (EDTA Tube)	Whole blood stimulation and cytokine concentration	Determine cytokine expression in circulation as well as compare cellular responses to previous studies
Green Top Sodium (Heparin Tube)	Flow cytometry analysis	Determine lymphocyte phenotype populations, granulocyte phagocytosis, lymphocyte activation (CD69 expression), and intracellular cytokines

Blood samples were collected via venipuncture into 7 mL vacutainers with sodium heparin or EDTA as the anticoagulant. Blood tubes were labeled with indelible marker with the soldiers' subject number and time of collection, placed in plastic test tube racks, sealed in a water tight plastic bag, and placed into an airline-approved insulated carrier for shipment. Samples were hand-delivered to the laboratory within 6 to 10 hours after blood draw. On receipt, blood samples were immediately assigned a sample number. Prenumbered labels were attached to each tube and a record was made linking the SFAS number with an identifying number. All whole blood samples were processed at room temperature. Standard biohazard class 2 and barrier precautions were observed at all times during the laboratory manipulations.

**LYMPHOCYTE BLASTOGENESIS** Responsiveness of lymphocytes (purified peripheral blood mononuclear cells) was determined (in a subset of subjects in each group, control n=29, treatment n=17) by a conventional method of mitogen stimulation (concanavalin A, 10 µg/mL). Cells were incubated for 72 hours after which cells were pulsed (18 hours before the end of incubation) with tritiated thymidine. [<sup>3</sup>H]Thymidine incorporation was measured in a scintillation counter. Response was determined by change from baseline levels.

**FLOW CYTOMETRY** The question of how immune cells regulate immune status and communicate with each other has been a focus of intense research for many years. As early as the 1960's immunologists first demonstrated that lymphocytes become activated *in vitro* in the presence of mitogens. Once lymphocytes become activated they pass through mitotic division and proliferation. One flaw of the above mentioned proliferation technique is that it does not allow the evaluation of responses by specific cell types. In recent years, research has focused on the soluble mediators (ie. interleukins and cytokines) which influence immune responses. There have also been advances in the identification of specific cell types. We used specific flow cytometric techniques to identify the percentage of individual lymphocyte types in the circulation as well as

the ability of specific lymphocytes to respond to mitogenic stimulation, as indicated by CD69 expression.

Flow cytometry, also known as flow microfluorimetry, was employed to identify the phenotype of lymphoid cell populations and subpopulations. This technique is used in diagnosis as well as monitoring disease states; for example, CD4<sup>+</sup> T lymphocytes are routinely quantitated as a prognostic indicator of HIV infection. Whole blood samples were incubated with monoclonal antibodies, with antibodies specific for a number of lymphocyte surface markers labeled with fluorochromes such as fluorescein isothiocyanate (FITC), phycoerythrin (PE) or peridinin chlorophyll protein (PerCP), after which red blood cells were lysed with lysing solution. The remaining cells were washed to remove cellular debris.

Lymphoid cell populations were initially classified by size and granularity (internal structure). Cell size was determined by measuring forward light scatter, and cell granularity was evaluated by measuring side light scatter. Cells that were larger (ie. monocytes) or more granular (ie. neutrophils) than lymphocytes were electronically excluded ("gated") so that the analysis focused on lymphocytes. Once fluorochrome labeled antibodies were attached to specific cells, the cells were passed through the path of a laser light, the fluorochrome absorbed light at one wavelength and fluoresced light at a different wavelength. By quantitating the fluorescence, the frequencies of specific cell types were determined.

This technology allowed rapid detection and enumeration of function and cell population. A variety of fluorochromes can be coupled to specific antibodies permitting the simultaneous analysis of multiple aspects of regulation and effector cell populations. Furthermore, we employed the technique of stimulating cells *in vitro* and then measuring activation markers and cytokine producing responsive lymphocytes.

Flow cytometric determination of the five-part differential white cell count in blood samples was determined by using the monoclonal antibody panel which follows:

	<u>Antibody Labeled with Specific Fluorochrome</u>			<u>Cell Types Detected</u>
	<u>FITC</u>	<u>PE</u>	<u>PerCP</u>	
CD16		CD14	CD45	Leukocyte differential count, gating
IgG1		IgG1	IgG1	Isotype controls
CD4		CD8	CD3	Helper, suppressor/cytotoxic T Cells
HLA-DR		p75(CD122)	CD3	Endogenously activated T-lymphocytes
CD16		CD56	CD3	Natural Killer (NK) lymphocytes
CD69		CD38	CD19&20	Endogenously activated B-lymphocytes

We also measured mitogen-stimulated activation by flow cytometry. Lymphocytes were activated with phytohemagglutinin (PHA) at 8 µg/ml and then analyzed for CD69 expression. PMA (phorbol-12-myristate-13-acetate) was also used as a stimulant.

Antibody combination:

<u>FITC</u>	<u>PMA Stimulated</u>	<u>PE</u>	<u>PERCP</u>
CD69	Yes/No	CD19&20	CD3

By this approach we were able to identify the population of lymphocytes that became activated (CD69 expression) for T- and B-lymphocytes. The population of cells that were CD3- or CD19&20 were considered Natural Killer cells.

To gain some insight into lymphocyte population shifts, during the second SFAS class we analyzed for memory and naive helper and suppressor/cytotoxic cells as well as granulocyte extravasation ( $10^{-8}$  M FMLP-stimulated and unstimulated cells) potential (expression of CD11b and L-selectin-method). Flow cytometric determination of T- and B-lymphocyte cooperation (second study class only) was indicated by CD40 ligand expression. The following monoclonals and stimulants were used.

<u>FITC</u>	<u>PE</u>	<u>PERCP</u>	<u>PMA and Ionomycin-Stimulated</u>
CD40L	CD69	CD3	Yes/No

Specific cell populations secreting cytokines were determined by flow cytometry.

<u>FITC</u>	<u>PE</u>	<u>PERCP</u>	<u>PMA and Ionomycin-Stimulated</u>
IFN	IL-4	CD4	No
IFN	IL-4	CD4	Yes
IgG <sub>1</sub>	IgG <sub>1</sub>	IgG <sub>1</sub>	Yes

Cell population data were initially determined using Becton-Dickson "Attractors" software in batch mode for Macintosh computers. Any sample outside of the normal range for a specific population was re-evaluated tube by tube, not as a panel batch mode.

**DELAYED TYPE SKIN HYPERSENSITIVITY (DTH)** *In vivo* immune status was assessed by administering a DTH skin test. This is an *in vivo* test of immune competency. A negative skin response (anergy) can indicate either no prior antigenic exposure or inability to respond to antigen. Furthermore, the lack of DTH response has been correlated with increased morbidity and mortality in a variety of clinical settings and disease states. "Cutaneous anergy may indicate functional impairment of, or abnormalities in, the cellular immune system. Delayed cellular hypersensitivity is a valuable measure of some type of immune response because it



involves a complex series of immunologic, cellular, mediator-associated, and vascular effects (Connaught Laboratories, Product information, page 2)." This type of test has been utilized for many years as a measure of immune status in malnourished patients (42, 52, 54). DTH skin tests (Multitest-CMI, Connaught Laboratories, Inc., Swiftwater, PA) were administered immediately after the blood draw at the end of the study.

The test kits contained a glycerin negative control and seven antigens of culture filtrates from the following microorganisms: *Clostridium tetani* (tetanus toxoid antigen), *Corynebacterium diphtheriae* (Diphtheria toxoid antigen), *Streptococcus* (Group C), *Mycobacterium tuberculosis* (C,D, and PN) plus *Mycobacterium bovis* (Vallee), *Candida albicans* (Candida antigen), *Trichophyton mentagrophytes* (trichophyton antigen), and *Proteus mirabilis* (Proteus antigen). A small amount of soluble antigen was introduced into the epidermis with the applicator. Theoretically, antigen specific circulating T-lymphocytes that have had prior exposure to the antigens, react by means of a specific antigen recognition which includes the interaction and communication of other cells which causes the release of cytokines and a mitotic response. The intensity of the response peaks between 24 and 72 hours after application. The test kit contained antigens that the soldiers would normally have been exposed to, particularly tetanus, and therefore anergy would indicate some level of immune dysfunction. All of the tests were administered by one individual to reduce the variability of administration. Tests were placed on the forearm after the area had been cleansed with alcohol. The pre-loaded applicator was pressed firmly and rocked back and forth and side to side to assure adequate pressure and uniform delivery of the antigens. After 36 hours, the tests were read for the largest induration or bump. Erythema without induration was not considered significant. An induration greater than 2 mm was considered as a positive response. Indurations were measured by one individual, once again to decrease the variability in the technique of reading the responses. Induration was measured (in mm), whenever a positive skin test was noted, measurements were made perpendicular and the largest response was recorded. All responses were noted and the total response was calculated by summing the total mm response. The following is a hypothetical example, "Three of the seven delayed hypersensitivity skin test antigens administered simultaneously produced induration reactions measuring 3 mm, 2 mm, and 5 mm respectively. The resulting score is 3/10mm. The number 3 represents the number of antigen yielding a positive response, while the 10 mm represents the sum total of induration from all three positive tests sites. If a patient is anergic the score is 0/0 mm signifying no reaction to any antigen and no resulting induration (Product insert, Connaught Laboratories, p 5)." The manufacturers of this device have noted that approximately 1.2% (n=315) of normal healthy male subjects are anergic while those who react have a mean total induration of 18.3 mm with a 4.5 mean number of positive responses (49).

All materials contaminated with blood products were disposed of appropriately in biological waste bags. Reagents used in chemistry procedures were collected in appropriate containers and sealed for proper disposal. Coordination was made with appropriate personnel at Womack Army Community Hospital, Fort Bragg, NC to dispose of the biological and chemical waste produced during the analyses.

**BODY COMPOSITION** Standing height (without boots) was measured while the soldier stood on a flat surface with the head positioned in the Frankfort Plane. Body weights were measured at baseline and end of study using a calibrated digital electronic battery powered scale accurate to 0.1 pound.

**STATISTICAL METHODOLOGY** This was a prospective, randomized, blinded, placebo controlled study (parallel design). There were 200 subjects that were randomized to participate in the study with 109 volunteers who completed the study. Three subjects did not have baseline white blood cell counts or lymphocyte percent values and were considered non-compliant in the study protocol. Therefore, these subjects were excluded from analysis. The remaining 106 subjects with complete data met the criteria of the protocol and were considered evaluable. The primary variables for this study included DTH reaction, complete white blood cell count and differential, lymphocyte phenotypes (percentage and absolute lymphocyte numbers), CD69 expression upon activation (percentage and absolute lymphocyte numbers), phagocytosis, CD11B/CD40 ligand expression upon activation (percent and absolute lymphocyte numbers), intracellular cytokine production by specific lymphocyte subsets (percentage and absolute lymphocyte numbers), and naive vs memory T-lymphocytes (percentage and absolute lymphocyte numbers). Blood samples were assessed at baseline and course completion (exit from SFAS for both control and treatment groups).

All analyses of continuous level data proceeded by first fitting a one way ANOVA model to the data and examining the residuals for meeting the assumption of being normally distributed. The data were not normally distributed (as evidenced by the Shapiro-Wilk test having  $P < 0.05$ ) in approximately 80% of the tests. Also, there were significant differences detected between the groups at baseline in a large number of tests. Therefore, the change was not analyzed but rather an analysis of covariance was used to analyze exit values using baseline values as the covariate. The analyses were all repeated with non-parametric methods utilizing rank data for the "baseline" ANOVA and normal scores for the "exit" covariate analysis (ANCOVA). Within groups, change scores (exit-baseline) were analyzed with a Sign Test. At exit, Spearman Correlation Coefficients were calculated for each variable response with total DTH to evaluate potential markers from the lymphocyte subsets. All results were considered to be statistically significant if the p value was less than 0.05.

## RESULTS

**COURSE COMPLETION AND BODY WEIGHT LOSS** There were 50 and 57 subjects who completed SFAS in the treatment and control groups, respectively. Reasons for dropping from the study were academic, physical fitness, voluntary withdrawal, and medical. There were no reported adverse reactions or aversions to either of the products tested.

	<u>Placebo Group</u>	<u>Treatment Group</u>
Total Subjects Enrolled	100	100
Administrative Drops	10	14



Medical Drops	7	7
Voluntary Withdrawal	14	16
Board Withdrawal	2	2
Voluntary Withdrawal with Injury	<u>10</u>	<u>11</u>
Total Completed	57	50*

\*One subject did not appear for final blood draw

There were no differences between the treatment and control groups regarding reason for dropping from the course. Body weight loss between groups was similar (Table 3) between groups and corresponded to weight loss found in previous studies.

Table 3. Body weight loss (pounds) of soldiers consuming control or treatment products and participating in SFAS (Mean +/- SEM)

	<u>Baseline Body Weight</u>	<u>End Body Weight</u>	<u>Body Weight Changes</u>
Control	177.0 +/- 2.3	170.6 +/- 2.1	-6.35 +/- 0.55
Treatment	175.7 +/- 2.5	169.4 +/- 2.2	-6.31 +/- 0.95

Dietary intake data is shown in Table 4. There were no significant differences in total daily intake of energy, protein, fat, carbohydrate, sodium, and cholesterol between groups, but, as anticipated, the treatment beverage provided significantly more vitamins and minerals than the control. Even in the control group most micronutrients were supplied in excess of the Military Recommended Daily Intake.

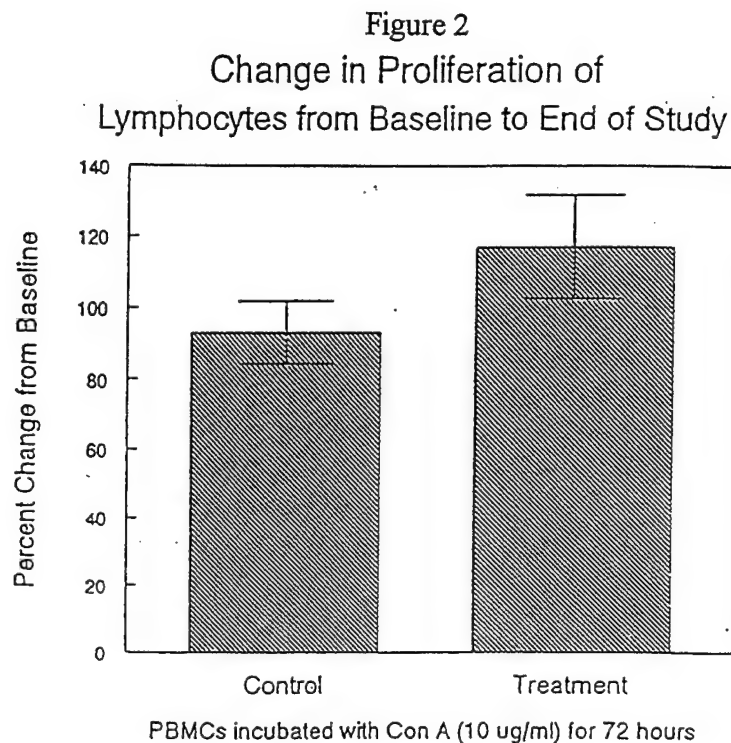
Table 4. Dietary Intake Means  $\pm$  Standard Deviations (percent of energy in parenthesis).

	Placebo n=57	Treatment n=50	MRDA <sup>#</sup>
Energy (Kcals)	3870 $\pm$ 378	3880 $\pm$ 430	
Protein (g)	140 $\pm$ 15 (14.4%)	139 $\pm$ 17 (14.3%)	100
Fat (g)	153 $\pm$ 18 (35.5%)	154 $\pm$ 22 (35.7%)	<35%
Carbohydrate (g)	495 $\pm$ 62 (51.2%)	500 $\pm$ 62 (51.6%)	
Vitamin C (mg)	277 $\pm$ 89*	767 $\pm$ 160	60
Thiamin (mg)	4.3 $\pm$ 0.8*	8.0 $\pm$ 0.8	1.6
Riboflavin (mg)	2.8 $\pm$ 0.5*	6.6 $\pm$ 0.5	1.9
Niacin (mg)	30 $\pm$ 4*	60 $\pm$ 4	21
Vitamin B <sub>6</sub> (mg)	4.3 $\pm$ 0.9*	8.0 $\pm$ 0.8	2.2
Folate (mcg)	287 $\pm$ 68*	924 $\pm$ 51	200
Vitamin B <sub>12</sub> (mcg)	4.9 $\pm$ 1.0*	18.5 $\pm$ 1.0	3
Vitamin A (RE)	2082 $\pm$ 57*	5174 $\pm$ 473	1,000
B-Carotene (RE)	276 $\pm$ 127*	2830 $\pm$ 186	UD
Vitamin E (IU)	7.2 $\pm$ 1.6*	267 $\pm$ 8.2	10
Calcium (mg)	1089 $\pm$ 248*	1423 $\pm$ 229	800-1,000
Phosphorus (mg)	2073 $\pm$ 317*	2233 $\pm$ 326	800-1,000
Magnesium (mg)	400 $\pm$ 57*	540 $\pm$ 54	350-400
Iron (mg)	19.5 $\pm$ 2.5*	27.9 $\pm$ 3.0	18
Zinc (mg)	16.7 $\pm$ 2.1*	65.2 $\pm$ 2.5	15
Potassium (mg)	4266 $\pm$ 685*	4730 $\pm$ 516	UD
Sodium (mg)	6675 $\pm$ 1565	6613 $\pm$ 1409	UD
Cholesterol (mg)	519 $\pm$ 98	538 $\pm$ 118	

<sup>#</sup>, Military Recommended Daily Allowance

\*, P < 0.05

**IMMUNOLOGIC MEASUREMENTS** Lymphocyte blastogenesis or proliferative capacity (response to mitogen, concanavalin A) of the lymphocytes, as determined by [ $^3\text{H}$ ]thymidine incorporation, showed an increased trend ( $P=0.1$ ) in the group which received the treatment product (Figure 2).



**DELAYED TYPE SKIN HYPERSENSITIVITY** There were fewer subjects in the treatment group (18%) vs. control group (39%) that were anergic to all antigens contained in Multitest CMI<sub>-</sub> (in normal non-stressed individuals there is a rate of 0-7% anergic) at the end of the course. Furthermore, there was a trend ( $P=0.07$ ) toward increased responsiveness (total induration mean  $\pm$  SEM millimeters) in the treatment (9.8  $\pm$  0.9 mm) versus control (7.8  $\pm$  1.0 mm) groups, respectively (in a normal non-stressed population a typical response is 18 mm).

#### **WHITE BLOOD CELL COUNTS, DIFFERENTIALS AND LYMPHOCYTE SUBSETS**

Generally, the results of white blood cell counts, differentials, and lymphocyte sub-sets are summarized below:

1. Total white blood cell numbers decrease over time in each group.
  - a. The pattern of decrease in the control group was a decrease in lymphocytes, monocytes and basophils with an increase in neutrophils and eosinophils.
  - b. The pattern of decrease in the treatment group is a decrease in lymphocytes, neutrophils, eosinophils, monocytes and basophils.
2. Eosinophils follow a similar pattern as neutrophils with a significant difference between control and treatment groups at the end of the study.

3. T-, B-, and NK lymphocytes decreased within both groups and there were differences between the two groups in the subsets of these lymphocyte populations.
  - a. There was a difference between groups in the subset of helper/inducer T-lymphocytes (CD4+/CD8-/CD3+); the lymphocyte numbers decreased in the control group while they were maintained in the treatment group.
  - b. The numbers of CD3+/CD56-/CD16+ decreased in the control group while they were maintained in the treatment group.
4. There were also changes in the populations of cells that were identified as either naive or memory cells (RA+/RO+/CD8+) lymphocytes.
  - a. The population identified as naive cytotoxic/suppressor lymphocytes (RA+/RO-/CD8+) was increased in the treatment group while there was a decrease in the control group.
5. There was a general pattern that the number of cells able to become activated (total CD69+) upon mitogen stimulation decreased within each of the groups (but increased lymphocyte proliferation, based upon tritiated [ $H^3$ ] thymidine incorporation, was found in the treatment group).
6. A shift from a Th1 profile to a Th2 cytokine production is more pronounced in the control group. The treatment group was similar to the baseline levels.
7. Continued evaluation of the data needs to be performed to make recommendations on what parameters should be monitored in future studies.

## DISCUSSION

There were dramatic changes in lymphocytes within both groups over time indicating that immune dysregulation had occurred during SFAS training. The novel nutritional product containing antioxidants, structured lipids, indigestible carbohydrates with vitamins and minerals (treatment product) partially attenuated the stress-induced immune dysregulation. Particularly prominent was the greater DTH responsiveness in the treatment group with fewer anergic soldiers compared to the control group. The control group had a similar rate of anergy to previous studies while the treatment group experienced a much lower rate, thereby potentially reducing their risk of infection. It was surprising that lymphocyte blastogenesis actually increased in the treatment group with suppression (as found in previous studies) in the control group. Subtle changes were apparent in the lymphocyte populations between groups. Although not as dramatic and definitive to indicate specific markers to monitor and definitively diagnose immune suppression, when combined with other markers of immune dysregulation, the results indicated that the treatment product helped maintain immune function under the stressful conditions of SFAS.

It is interesting that while investigating how nutritional status influences immune function (using the same Multitest CMI used in this study), previous investigators correlated responses to DTH with the incidence of respiratory tract infections (93). These investigators found that anergic children had 20% higher risk of respiratory tract infection than the immunocompetent children. In another pediatric study, researchers in Peru administered the Multitest CMI test and then followed the subjects for acute respiratory infections, gastrointestinal infections and episodes

of diarrhea for six months after the test was administered. Responsiveness to DTH was inversely related to incidence of respiratory infection and diarrhea (8). They found that, "...anergic children experience a 20 per cent higher attack rate than immunocompetent children." This supports the idea that DTH responsiveness can be a predictor of susceptibility to common respiratory and enteric infections.

Multitest CMI tests have also been used to evaluate immune responsiveness in adult cancer patients. It was found that patients with localized cancer had an anergy rate of 14.8% while those with disseminated cancer rose to 22.3% (49). Other investigators have tried to determine whether high rates of DTH anergy were associated with the stage of the cancer or the effect of undernutrition. Burke et al. (11) found that cancer patients in general had significantly lower DTH responses, but there were no significant differences between benign and malignant groups of equivalent nutritional status. They concluded that cell mediated immunity as measured by DTH in gastrointestinal cancer was due to undernutrition and was not a cancer specific effect. Burke's results were confirmed in another study where protein-energy malnutrition depressed cell-mediated immunity but not malignant tumors (74). Higher anergy rates in soldiers experiencing intense training may suggest increased risk of infection. Our study suggests that this may be partially reversed by nutritional intervention.

Infection rates are another important indicator of immune status. Although it was not the primary focus of this study to quantitate the infection rates in the subjects who participated (particularly since this was a small sampling), effort was made to obtain this information. Army physicians and physician assistants noted symptoms (i.e. fever) and physical exam findings and medical records were reviewed blinded. Our findings demonstrate that there were more upper respiratory tract infections in the control group as compared to the treatment group (12% vs 7%).

The specific cause of the immune dysregulation and suppression during SFAS appears to be the combination of sleep deprivation, strenuous physical exertion, emotional stress, and nutritional deprivation. This training approximates the stress of combat. In this study we manipulated only one variable, nutrition, and found that it attenuated some of the immunologic changes. We also attempted to overcome the caloric deficit by providing an energy dense nutritional product. It was interesting that by simply providing more energy, soldiers adjusted their food intake and weight loss was not minimized. This observation also suggests that there may be specific nutrients that can be fed to an individual experiencing an energy deficit that can minimize or prevent immune suppression.

Many diseases involve the immune system and or free radical reactions. Free radicals may be formed during normal cellular, organ and systemic function, and their harmful effects are associated with cellular disruption or damage. Furthermore, this damage can also affect proteins, membranes, and DNA. Many functions of immune cells require membrane fluidity which can depend on fatty acid composition. As the fatty acid composition changes, membrane fluidity also varies, but, most importantly, as membrane incorporation of polyunsaturated lipids occurs, so does the potential for lipid peroxidation increase. Mice fed oxidized lipids had a smaller thymus,

increased T-cell dysfunction, and reduced membrane fluidity, all associated with lymphocyte inability to respond to challenge (6). Since there is ample evidence that antioxidants as well as oxidative stress can play a role in immune function, a number of antioxidants were included in the treatment product.

Other studies have found an increased risk of respiratory infections in subjects performing heavy physical or strenuous exercise. In a study evaluating vitamin C supplementation, a group of runners were evaluated for upper respiratory infections (70). The runners were given a daily supplement of 600 mg vitamin C or placebo for 3 weeks prior to an event, and upper respiratory tract infection rates were quantified after participation in a competitive ultramarathon race. They found that 68% of the subjects in the placebo group experienced an upper respiratory tract infection while only 33% of the subjects experienced infection in the vitamin C group ( $P < 0.01$ ). The investigators also found that the duration and severity of symptoms from infections were significantly less in the group that consumed vitamin C. While the subjects in this study received 500 mg daily, it is not possible to identify the nutritional component(s) responsible for the specific results observed in this study. Recently, Hemila (36) summarized three studies in which vitamin C was supplemented in subjects under acute physical stress and concluded that vitamin C supplementation seems to support immune function and may be beneficial for subjects performing intense exercise who may have problems with frequent respiratory infections.

During the course of this study, a significant decrease in the total number of white blood cells was observed. The immune status profile of the control group was different from the treatment group. The subjects in the control group experienced a 6% decrease in white blood cells which was primarily a drop in the lymphocyte number (-20%), with increases in neutrophils (7%) and eosinophils (20%). Neutrophils are a component of the first wave of cellular defenses and it has been shown that the percent of neutrophils increases following strenuous exercise, e.g. marathons (10). Further, a dramatic rise in neutrophil concentration upon infection as a response to invading organisms is commonly observed. An inability of the neutrophils to migrate to loci of infection may be sufficient to open a window of opportunity for infection (46). The group that received the treatment product had a reduction in WBC but did not have an increase in neutrophils and actually had a -4.7 and -18 percent reduction in the number of circulating neutrophils and eosinophils, respectively. The rise in neutrophil concentration in the control group is consistent with the increased infection rate as compared to the treatment group. We also found that the subjects who consumed the treatment formula experienced enhanced neutrophil phagocytosis (+27.5%), while there was no change in the group consuming the control formula thus indicating improvement in neutrophil function.

Preventing infectious diseases has been a priority for all branches of the military yet, even during recent military conflicts (Operation Desert Shield), there have been epidemics of infectious diarrhea despite efforts to secure a safe supply of food and water. These epidemics may potentially compromise the capabilities of a military force during critical periods (39). Since this formula provided immunologic benefit to soldiers under a simulated operational scenario, this

treatment formula provides a basis which could be developed to evaluate other nutritional components that affect immune function in highly stressed populations.

An understanding of the immunologic changes induced by chronic stress provides an opportunity to study mechanisms which lead to a breakdown in immunity and an increased susceptibility to infection as well as how nutritional supplements may combat such immune dysregulation. This study is the initial report demonstrating a beneficial effect of a structured nutritional supplement on stress-altered immune function. We have demonstrated that a nutritional supplement may attenuate biochemical markers of immune dysregulation, enhance skin DTH response, and reduce susceptibility to acute upper respiratory tract infections.

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